Comparative Genomic Hybridization of Formalin-fixed, Paraffin-embedded Breast Tumors Reveals Different Patterns of Chromosomal Gains and Losses in Fibroadenomas and Diploid and Aneuploid Carcinomas[1]

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ABSTRACT

Comparative genomic hybridization serves as a screening test for regions of copy number changes in tumor genomes. We have applied the technique to map DNA gains and losses in 33 cases of formalin-fixed, paraffin-embedded primary breast tumors (13 fibroadenomas and 10 diploid and 10 aneuploid carcinomas). No genomic imbalances were found in fibroadenomas. Recurrent findings in adenocarcinomas include copy number increases for chromosomes 1q (14 of 20 samples), 8q (10 of 20), 17q (5 of 20), 6p (3 of 20), 13q (3 of 20), and 16p (3 of 20), and copy number decreases for chromosomes 22 (7 of 20), 17p (6 of 20), and 20 (3 of 20). Regional high level copy number increases were observed on chromosome bands 1q32, 8p11, 8q24, 10p, 11q13, 12p, 12q15, 17q11–12, and 17q22–24. The majority of the samples were studied for gene amplification of c-*myc*, c-*erb*B2, *cyc*D1, and *int*-2 by means of Southern blot analysis. The comparison with DNA ploidy measurements revealed a different distribution and a significantly higher number of chromosomal aberrations in aneuploid tumors than in diploid tumors and in fibroadenomas.

INTRODUCTION

For 50 years, breast cancer, with an incidence of 90 in 100,000, has had the highest cancer mortality among women in the Western world (1). Screening tests and diagnostic improvements over the recent years did not result in an overall increased survival time. This is mainly attributable to the lack of parameters for individual therapy adaptation, because prognostic parameters are still defined through lymph node status, tumor size, and histopathological grading. All these parameters, however, describe the tumor state rather than the tumor aggressiveness.

Measurements of DNA content on formalin-fixed tumor samples in a number of prospective and retrospective studies revealed that aneuploid tumors follow a more malignant course than do predominantly diploid tumors (2–4). Benign fibroadenomas of the breast reveal exclusively diploid or tetraploid DNA distributions. DNA ploidy measurements, however, do not provide any information on the chromosomal origin of genomic imbalances, which would be an important step toward a molecular analysis of genetic alterations (5).

CGH[4] is a new molecular cytogenetic technique that is based on quantitative two-color fluorescence *in situ* hybridization (6–8). CGH allows, in a single experiment, the detection of genetic imbalances in solid tumors or any desired test genome and the determination of the chromosomal map position of gains and losses of chromosomes or chromosomal subregions on normal reference metaphase preparations. CGH has been applied for the detection of chromosomal aberrations in a variety of solid tumors, including breast and lung cancers and astrocytic and renal carcinomas (9–12). A distinct advantage of CGH compared with other comprehensive approaches to detect genetic aberrations in tumors, e.g., karyotyping, is provided by the fact that genomic DNA is the only source required from the tumor specimen, because genetic imbalances are identified on reference chromosomes. Thus, DNA extracted from an archived tumor specimen can be used as well (13).

In the study presented here, we have applied CGH to a series of 13 fibroadenomas of the breast and 10 diploid and 10 aneuploid breast carcinomas. All the samples investigated were analyzed previously for DNA content, and the majority of the samples were analyzed for the amplification of protooncogenes, *p53* expression, and c-*erb*B-2 expression. The clinical follow-up was recorded. In all cases, the DNA was extracted from formalin-fixed, paraffin-embedded tissue sections.

No aberrations were detected in the series of fibroadenomas studied here. Regarding the carcinomas, recurrent regions of increased copy numbers were observed most frequently on chromosomes 1q, 8q, and 17q22–24. Consensus regions of decreased copy number mapped to chromosomes 17p and 22. Eight chromosomes were subject to copy number changes in diploid tumors. A significantly higher number of genetic alterations, including high-level copy number increases, were detected in aneuploid tumors on 19 different chromosomes.

MATERIALS AND METHODS

Patient Material. In the present study, surgical specimens from 13 fibroadenomas and 20 primary adenocarcinomas of the female mammary gland were analyzed. The histopathological classification was based on the WHO histological typing of breast tumors (14). Nineteen carcinomas were the ductal type, and one was the lobular type (case 6; Table 1).

Metaphase Chromosome Preparations. Metaphase chromosome spreads were prepared following standard procedures from peripheral blood lymphocytes from a healthy donor. The preparations were chosen following the criteria defined by du Manoir et al. (15).

Probe DNA Preparation. Normal control DNA was prepared from peripheral blood lymphocytes of a healthy male donor as described (16). Formalin-fixed, paraffin-embedded tumor samples were provided in 50-μm slices. Prior to DNA extraction, the paraffin was removed manually as completely as possible. The tissue was cut in small pieces, and remainders of paraffin were removed by incubation in xylene (45°C for 15 min), followed by one washing step in ethanol. After centrifugation, the samples were dried in a Speed Vac (Savant). The samples were resuspended in 1 ml sodium isothiocyanate (1 M) and incubated at 37°C, overnight. The tissue was washed and resuspended in 400 μl DNA isolation buffer (75 mM NaCl, 25 mM EDTA, and 0.5% Tween 20). Proteinase K was added to a final concentration of 1 mg/ml, and the tissue was incubated overnight at 55°C. The DNA was then subjected to two phenol extraction steps, one wash in chloroform:isoamylalcohol [24:1 (v/v)], ethanol precipitated, and resuspended in sterile water.

Table 1 *Comparison of the results from DNA ploidy measurements, protooncogene amplification as detected by Southern blot analysis, and CGH in 20 cases of breast carcinoma[a]*

Case	Cytometry DNA ploidy	Southern blot Amp	CGH analysis		
			Gains	Losses	Amp
1	pD[b]	—	1q, 5p	17p	—
2	D	—	1q, 8	17p, 20, 22q	
3	D	—	8q	—	—
4	D	----	1q	—	
5	D	cycD1	1q	17p	12q15
6	D	—	1q	22q	—
7	D	—	1q	20q, 22q	—
8	D	---	1q	3p	----
9	D	n.a.	1q	—	—
10	D	n.a.	1q, 8q, 17q23-24	---	
11[c,d]	A	c-erbB2, c-myc	1p11-12, 1q, 4p, 8q, 16p, 18p	15q, 22q	10p, 12p
12[c]	A	c-erbB2	7q, 8q	8p, 17p	8q23-24, 8p11.2, 11q13-14, 17q11.2-12, 17q22-24
13	A	cycD1, int-2	1q32-44, 2q24-31, 4q21, 11q13-14, 12q15-24, 13q32-33, 20q11-13.2	14q31-32, 20p12-13, 22q13	8q23-24, 17q22-25
14[d]	A	int-2	2q, 8q	12q24 18, 22q	—
15	A	---	1p31-32, 1q, 2, 4, 5p, 6p, 8q	17p	17q22-24
16	A	—	4q21, 8q, 11p 13q22-34	16, 22q	
17	A	—	8q, 13q31-34	12q, 17p	8q24
18	A	n.a.	3q, 7, 15q14-26	3p	—
19	A	n.a.	6p, 16p	6q	1q32
20	A	n.a.	1q, 6p21.2-25, 20q	—	17q11.2-12

[a] Note that the CGH analysis reveals a higher number of DNA gains and losses, including amplifications, in aneuploid tumors.
[b] pD, proliferating diploid tumor; D, diploid tumor; A, aneuploid tumor; —, amplications or copy number changes not observed; n.a., not analyzed.
[c] c-erbB-2 expression positive.
[d] Mutant p53 expression positive.

Labeling and Fluorescence *in Situ* Hybridization. Normal male DNA was labeled in a standard nick translation reaction substituting dTTP by digoxigenin-11-dUTP (Boehringer Mannheim, Indianapolis, IN); tumor DNA was labeled by substituting dTTP by biotin-16-dUTP (Boehringer Mannheim). The DNase I concentration in the labeling reaction was adjusted to reveal an average fragment size of 1000 bp. Prior to fluorescence *in situ* hybridization, the labeled probes were separated from unincorporated nucleotides by Sephadex columns (17).

For CGH, 200 ng normal, digoxigenin-labeled DNA and 200 ng biotin-labeled tumor DNA were ethanol precipitated in the presence of 10 μg salmon sperm DNA and 30 μg Cot-1 fraction of human DNA (GIBCO-BRL, Gaithersburg, MD). The probe mixture was dried and resuspended in 10 μl hybridization solution (50% formamide, 2× SSC, and 10% dextran sulfate). The DNA was denatured at 76°C for 5 min and allowed to preanneal at 37°C for 1 h. The metaphase preparations were denatured at 80°C for 2 min in 70% deionized formamide and 2× SSC and dehydrated through an ethanol series (70, 90, and 100%). The probe mixture was applied to the denatured metaphase chromosomes under a coverslip (18 mm²), sealed with rubber cement, and hybridized for 4 days at 37°C.

Posthybridization steps were performed as described in detail (17). The biotinylated sequences were detected with FITC conjugated to avidin (Vector Laboratories, Inc., Burlingame, CA). Probe sequences haptenized with digoxigenin were visualized with antidigoxigenin Fab fragments conjugated to rhodamine. Chromosomes were counterstained with DAPI and embedded in an antifading agent to reduce photobleaching.

Microscopy and Digital Image Analysis. Gray level images were acquired for each fluorochrome with a cooled charge-coupled device camera (Photometrics, Tucson, AZ) coupled to a Zeiss Axiophot or a Leica DMRBE epifluorescence microscope. Chromosomes were identified using DAPI banding. Fluorescence ratio images were calculated as described; the ratio profiles of individual reference chromosomes were determined by a custom computer program (15) and run on a Macintosh Quadra 950. Briefly, after the determination of the chromosomal axis for each chromosome in every metaphase, individual FITC and rhodamine profiles were calculated. These were used for the computation of the ratio (FITC:rhodamine) profiles (Fig. 2, *B* and *D*). The three vertical lines on the right side of the chromosome ideogram represent different values of the fluorescence ratios between the tumor and the normal DNA. The values are 0.75, 1, and 1.25 from left to right, respectively. These values were chosen as thresholds for the identification of DNA copy number decreases (below 0.75) and increases (above 1.25) as described (15). The curve

shows the ratio profiles that were computed as mean values of at least five metaphase spreads.

Southern Blot Analysis. DNA was isolated from a sample of approximately 0.05–0.4 g removed from the tumor during pathological investigation directly after surgery following standard preparation techniques. The DNA was restriction digested, electrophoresed, and transferred to a nylon blotting membrane in 0.4 M NaOH. The membranes were then rinsed in 2× SSC, vacuum baked for 2 h at 80°C, and washed in 2× saline-sodium phosphate-EDTA and 1% SDS for 2 h at 65°C.

Hybridization was performed in 4× saline-sodium phosphate-EDTA, 5% dextran sulfate, 1% SDS, 5 mg/ml human genomic DNA, and 200 mg/ml herring sperm DNA (Sigma Chemical Co., St. Louis, MO) at 70°C overnight. The probe concentration was 0.3–5.0 ng/ml buffer. After stringency washes, the filters were rinsed in 2× SSC at room temperature and exposed for autoradiography using Kodak X-Omat AR XAR5 film. Signal intensity was quantified densitometrically.

For each protooncogene, a nonamplified gene on the same chromosome was cohybridized. The control probes were located on the same chromosomes as the genes under study to avoid obtaining false-positive amplification results in cases in which chromosome copy numbers were increased. Protooncogenes were considered amplified if the ratio between the band strengths of the probe for the protooncogene and the chromosome-specific control probe was at least twice as high as the ratio for the lymphocyte DNA. The c-*myc* probe was the 1.2-bp pRyc 7.4 fraction cloned in the *Pst*-1 site of pBR322. The chromosome-specific control for c-*myc* was a DNA clone that maps to chromosomal band 8q22. The probe for human *int*-2 was Hu-3 (0.9 kbp) cloned in the *Sac*I site of pSP64. The chromosome-specific probe for *int*-2 was a 2.2-kilobase pair apoA1 fragment cloned in the *Pst*-1 site of pUC8. The c-*erb*B-2 and *cyc*D1 probes were prepared using PCR, as were the chromosome-specific probes for c-*erb*B-2 and c-*myc* (GP11b). Probes were labeled to a specific activity of approximately 10^9 cpm/μg by random priming (18).

Immunohistochemistry. Immunohistochemical staining was performed using an avidin-biotin immunoperoxidase complex technique. Tissue sections were incubated with a polyclonal antibody against the cytoplasmic domain of the human c-*erb*B-2 raised in sheep (OA-11–854; Cambridge Research Biochemicals, Cambridge, United Kingdom). For *p53* analysis, the sections were incubated overnight with the primary polyclonal antibody CM-1 (Novocastra Laboratories, Newcastle upon Tyne, United Kingdom). Biotinylated rabbit antisheep (c-*cerb*B-2) or antirabbit (*p53*) affinity-purified antibody (BA-6000; Vector) was used. After light counterstaining with hematoxylin, the slides were

dehydrated and mounted. Concerning c-cerbB-2 evaluation, a known case of carcinoma *in situ* of the comedo type served as a positive control. Negative controls were sections of the same case incubated with inactivated sheep serum instead of the primary antibody. Concerning *p53* evaluation, only cases with cells showing a distinct nuclear reaction were regarded as *p53* mutant positive.

Cytometric DNA Assessments. Feulgen-stained fine-needle aspirates from each tumor were evaluated on an image analysis system (Ahrens System, Bargteheide/Hamburg, Germany), using a microscope (Nikon, Tokyo, Japan; plan objective, 40/0.95), equipped with a video charge-coupled device camera (Panasonic WV-CD 20; Matsushita Electric Trading Co., Osaka, Japan) that was interfaced with an IBM-compatible data unit (Victor V386; Victor AB, Stockholm, Sweden). The Feulgen-staining procedure, internal standardization, and tumor cell selection were based on methods described previously (19). The DNA histograms were recorded as "diploid" when a single distinct peak containing >90% of the cell counts was present in the G_0-G_1 region of the normal cell population (DNA index, 0.9–1.1). The remaining fraction of cells showed DNA values in the tetraploid region. The term "proliferating diploid" is used to classify tumors that reveal a distinct diploid peak and a minor tetraploid peak (<20% of the total cell population) and 5–20% of the cells in the S-phase of the cell cycle. "Aneuploid" DNA profiles were characterized by values located outside the normal diploid and tetraploid areas with more than 10% of the cells exhibiting DNA values exceeding 5c. Examples of the histograms are presented for a diploid carcinoma (case 1) and an aneuploid carcinoma (case 12; Fig. 1, *A* and *B*).

RESULTS

This study reports the results of CGH analysis from DNA extracted from 13 fibroadenomas of the breast and 20 cases of carcinomas of the mammary gland. In all instances, the material was formalin fixed and paraffin embedded. The findings were compared with DNA content measurements, Southern blot analysis of oncogene amplification, and immunohistochemistry. Clinical follow-up data were available for all cases.

Fibroadenomas are common benign lesions of the mammary gland originating from epithelial and stromal cells (20, 21). The tumors grow encapsulated and never metastasize. Image cytometry reveals invariably diploid DNA contents, and oncogene amplifications are not detected.

The adenocarcinomas investigated in this study were confined to two groups: (*a*) tumors that are diploid; and (*b*) tumors that reveal an aneuploid DNA content. Representative examples of the DNA histograms are displayed in Fig. 1.

Examples of a CGH analysis of DNA extracted from paraffin sections of a fibroadenoma and an aneuploid ductal breast carcinoma are presented in Fig. 2. A homogeneous labeling of all chromosomes is visible in the fibroadenoma case, as reflected by the ratio image (Fig. 2*A*). The average ratio profile is consistently close to the central line (Fig. 2*B*). The results with DNA extracted from an aneuploid carcinoma (case 13) are presented in Fig. 2, *C* and *D*. Blue indicates balanced copy numbers; regions of increased copy numbers appear green; and red indicates regions that are underrepresented in the tumor genome. Based on a quantitative analysis of the fluorescence values

for each fluorochrome, mean ratio profiles were calculated that reflect copy number changes in the tumor genome and allow for a chromosomal band assignment of genetic imbalances (15). In case 13, regions of decreased copy numbers were mapped to chromosomes 14q31–32, 20p12–13, and 22q13; regions of increased copy numbers include chromosomes 1q32–44, 2q24–31, 4q21, 8q23–24, 11q13–14, 12q15–24.2, 13q32–34, 17q22–25, and 20q11–13.2. The apparent loss of sequences at the tip of the short arm of chromosome 1 in this case was not included in the summary of DNA copy number changes (see Fig. 5), because this chromosomal region was subject to copy number changes in control experiments as well. Mean fluorescence ratio profiles were used to determine copy number changes in all cases presented in this study. When high copy number changes were detected, the hybridization was repeated on less-condensed metaphase chromosome preparations to determine the chromosomal mapping position more precisely. An example that shows an amplification on chromosome 12q15 (case 5) is presented pictorially in Fig. 3, which presents a merged image of the tumor-specific green fluorescence on DAPI-stained chromosomes. The arbitrarily defined threshold allows the visualization of high-level copy number increases relative to chromosomal bands.

Thirteen fibroadenomas were analyzed. None of the tumors revealed a DNA copy number variation detectable by CGH. All fibroadenomas were diploid, and no amplification of protooncogenes was evident by means of Southern blot analysis. Increased *p53* expression was not observed.

Ten tumors (cases 1–10) revealed diploid patterns of DNA content. None of the diploid tumors showed an expression of c-*erb*B-2 or mutant p53 protein. In one case, the amplification of the *cyc*D1 gene (which maps to chromosomal band 11q13) was detected by Southern blot analysis (case 5; Table 1). In this tumor, however, a high-level copy number increase was obvious only on chromosomal band 12q15 after CGH (Fig. 3). The most consistent finding in diploid tumors was an overrepresentation of chromosomal arm 1q (9 of 10 cases). In one of the diploid tumors (case 10), the gain of chromosome 1q exceeded clearly the value for a trisomy. In three cases, chromosome 8q showed increased copy numbers. Decreased fluorescence ratios were observed for chromosomal arms 17p and 22q in three cases, indicating a loss of genetic material. Copy number changes in the diploid group involve either entire chromosomes or chromosomal arms. The results are summarized in Fig. 4.

Ten tumors were aneuploid according to the classification established by Auer *et al.* (19). In this fraction, the amplification of c-*erb*B-2, c-*myc*, *cyc*D1, and *int*-2 was detected in four tumors by Southern blot analysis (Table 1). The tumors that revealed DNA amplification of the c-*erb*B-2 oncogene were also positive for c-*erb*B-2 expression, as demonstrated by immunohistochemistry. Mutant *p53* expression was observed in two cases. For a summary, see Table 1. CGH analysis revealed recurrent DNA gains on chromosomes 8q (7 of 10 cases), 1q (5 of 10), and 13q (3 of 10). High-level

Fig. 1. Representative examples of DNA histograms that were used for the classification of the tumors used in this study as diploid (*A*) or aneuploid (*B*). Note the narrow peak at 2c in the diploid tumors and the scattered DNA values in the aneuploid tumors.
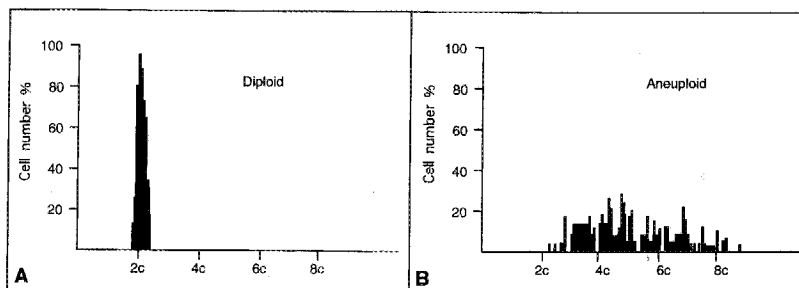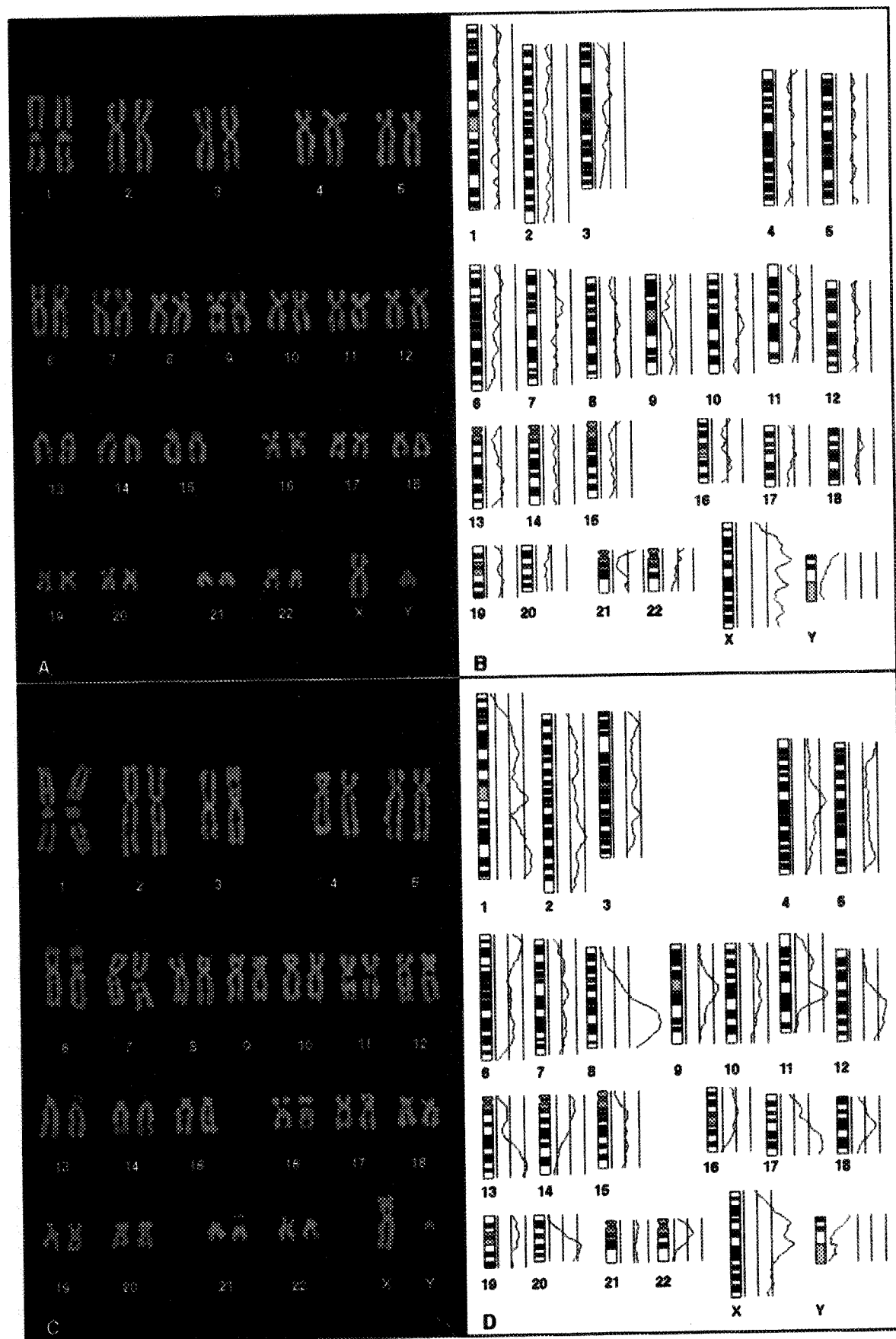
Fig. 2. Examples of the CGH analysis of a case of a fibroadenoma (A and B) and an aneuploid mammary adenocarcinoma (case 13; C and D). The ratio images are displayed in A and C. Regions of balanced copy numbers appear *blue*, whereas *red* indicates loss of sequences in the tumor genome, and *green* indicates gain of sequences in the tumor genome. Note the consistency of the painting pattern on the homologous chromosomes. The average ratio profiles are displayed in B and D. The three *vertical lines* on the *right* of the chromosome ideograms represent different values of the fluorescence intensities between the tumor and the reference genome. The values are 0.75, 1, and 1.25 from *left* to *right*, respectively. The ratio profile was calculated as a mean of at least five metaphase spreads.
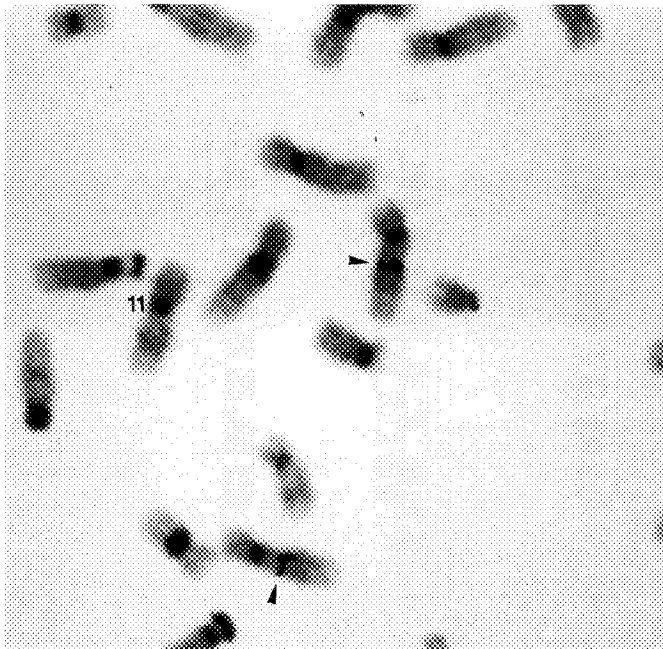
Fig. 3. Example of the chromosomal mapping position of an amplification site in a diploid breast carcinoma (case 5). The CGH experiment was performed on less-condensed chromosomes. The threshold was chosen arbitrarily to visualize the highest FITC signal intensity on DAPI-banded metaphase chromosomes. The signals are observed at chromosome band 12q15 (*arrowheads*). Chromosome 11 revealed no signals, although an amplification of the *cyc*D1 oncogene was detected by Southern blot analysis.

DNA copy number increases were mapped to chromosomal bands 8q24 (3 of 10), 17q22–24 (3 of 10), 17q11–12 (2 of 10), 11q13 (1 of 10), 1q32 (1 of 10), 8p11 (1 of 10), 10p (1 of 10), and 12p (1 of 10). Frequent DNA losses were mapped to chromosomes 22q (4 of 10), 17p (3 of 10), and 12q (2 of 10). The results are summarized in Fig. 5. In this subset of aneuploid tumors, copy number changes involved not only entire chromosomes or chromosomal arms but also chromosomal subregions. Consistent findings between CGH and Southern blot analysis of the amplification of protooncogenes were made in case 11 for the c-*myc* oncogene (located on chromosomal band 8q24), in case 12 for the c-*erb*B-2 oncogene (17q11.2–12), and in case 13 regarding the *cyc*D1 and *int*-2 protooncogenes (11q13). The amplification of the c-*erb*B-2 oncogene in case 11 and the amplification of the *int*-2 gene in case 14, as analyzed by Southern blot analysis, were not accompanied by increased fluorescence ratios at the respective chromosomal bands after CGH analysis. Amplification of c-*myc* was not observed in cases 12, 13, and 17, in which increased ratios on chromosome 8q or chromosomal band 8q24 were detected after CGH.

DISCUSSION

The characterization of prognostic predictors is one of the most important needs for individual therapy adaptation in patients suffering from breast tumors. This is attributable to the marked variability of the clinical course and to the broad spectrum of therapeutic alternatives that range from restricted surgical resection to radical mastectomy with adjuvant chemotherapy and radiation (1).

In addition to clinical variables, *i.e.*, lymph node status and tumor size, several parameters were defined that, indeed, correlate with the clinical course. Those include the amplification of the c-*erb*B-2 protooncogene (22, 23), the estrogen receptor status (24), and defined mutations of tumor suppressor genes, *e.g.*, *p53*, and mutant p53 protein overexpression (25, 26). Another potent predictor of the clinical course is provided by DNA content measurements. With the use of Cox multivariate analyses, nuclear DNA content was found to

provide significant prognostic information additional to that given by all other clinical and histomorphological variables taken together (27).

To investigate whether benign breast diseases and diploid carcinomas reveal recurrent chromosomal aberrations and to determine the chromosomal map position of genomic regions that are subject to copy number changes in aneuploid cancers, we have analyzed a series of 13 fibroadenomas and 20 primary breast carcinomas using CGH. The DNA was extracted from archived, formalin-fixed specimens. The possibility of using paraffin-embedded material is a crucial improvement compared with other comprehensive approaches to determine copy number changes in tumor genomes, *i.e.*, chromosomal banding studies, because it allows retrospective analysis of tumor collections in pathology archives. In addition to the assessment of DNA ploidy in this collection of tumors, the amplification of the protooncogenes c-*myc*, *cyc*D1, *int*-2, and c-*erb*B-2 was investigated. Moreover, immunocytochemistry was performed to analyze the samples for c-*erb*B-2 expression and mutant *p53* expression.

Fibroadenomas are biphasic, benign tumors that consist of cells of stromal and epithelial origin. Only few studies using karyotype analysis report clonal aberrations in fibroadenomas (28–30). Individual clones were present in 20% of all cases studied and revealed the involvement of chromosomes 8, 11, and 12. A trisomy for chromosomes 8 and 11 was confirmed by interphase cytogenetics; however, only 2–15% of the cells revealed the specific aberration (29). It is still disputed whether fibroadenomas represent a polyclonal hyperplasia rather than a monoclonal precancerous lesion (20, 31). Our findings support the first hypothesis. The failure to detect any DNA copy number changes in fibroadenomas indicate either: (*a*) that, indeed, copy number changes are not present in these tumors and that chromosomal aberrations detected by karyotype analysis are balanced; or (*b*) that the polyclonal character prevents the identification of the changes due to a low number of cells actually carrying a specific aberration (which would not be detectable by CGH). Further studies using a combination of microdissection of multiple defined regions of these tumors and subsequent CGH analysis (13) will allow us to address this problem. It might also be interesting to analyze the few cases that are known to have progressed to carcinoma (32).

In contrast to fibroadenomas, diploid carcinomas revealed consistent chromosomal aberrations. However, the number of genetic changes observed in diploid tumors was considerably lower compared with aneuploid tumors. Eight chromosomes were subject to copy number changes in diploid carcinomas, with an average number of 2.4 aberrations/case. An amplification was observed only once. In aneuploid carcinomas, however, 19 chromosomes were involved in alterations; the average number of aberrations per case increased to 6.8. Thirteen amplifications were mapped.

The most consistent aberration in diploid carcinomas was a trisomy of the long arm of chromosome 1 (9 of 10), followed by trisomy for chromosome 8q (3 of 10). Monosomy was detected for chromosomes 17p (3 of 10) and 22q (3 of 10). The high percentage of chromosome 1q overrepresentation is in accordance with data published using karyotyping (33–35). Overrepresentation of 1q was also observed by Kallioniemi *et al.* (9), using CGH, and Muleris *et al.* (36), using a modified CGH technique to study DNA gains in primary breast carcinomas and tumor cell lines. The second frequent trisomy involves the long arm of chromosome 8, which harbors the c-*myc* oncogene on chromosomal band 8q24. High-level amplifications of c-*myc*, however, are not detectable in this series of diploid tumors, which is in accordance with data from Southern blot analysis. Regions of common loss in the diploid tumors include chromosomal arms 17p and 22q. The tumor suppressor gene *p53* is localized on chromosomal band 17p13.1. Deletions of this tumor suppressor gene are a frequent finding in breast (and other) carcinomas (37). A second frequently monosomic chromosomal segment comprises 22q. This chromosomal
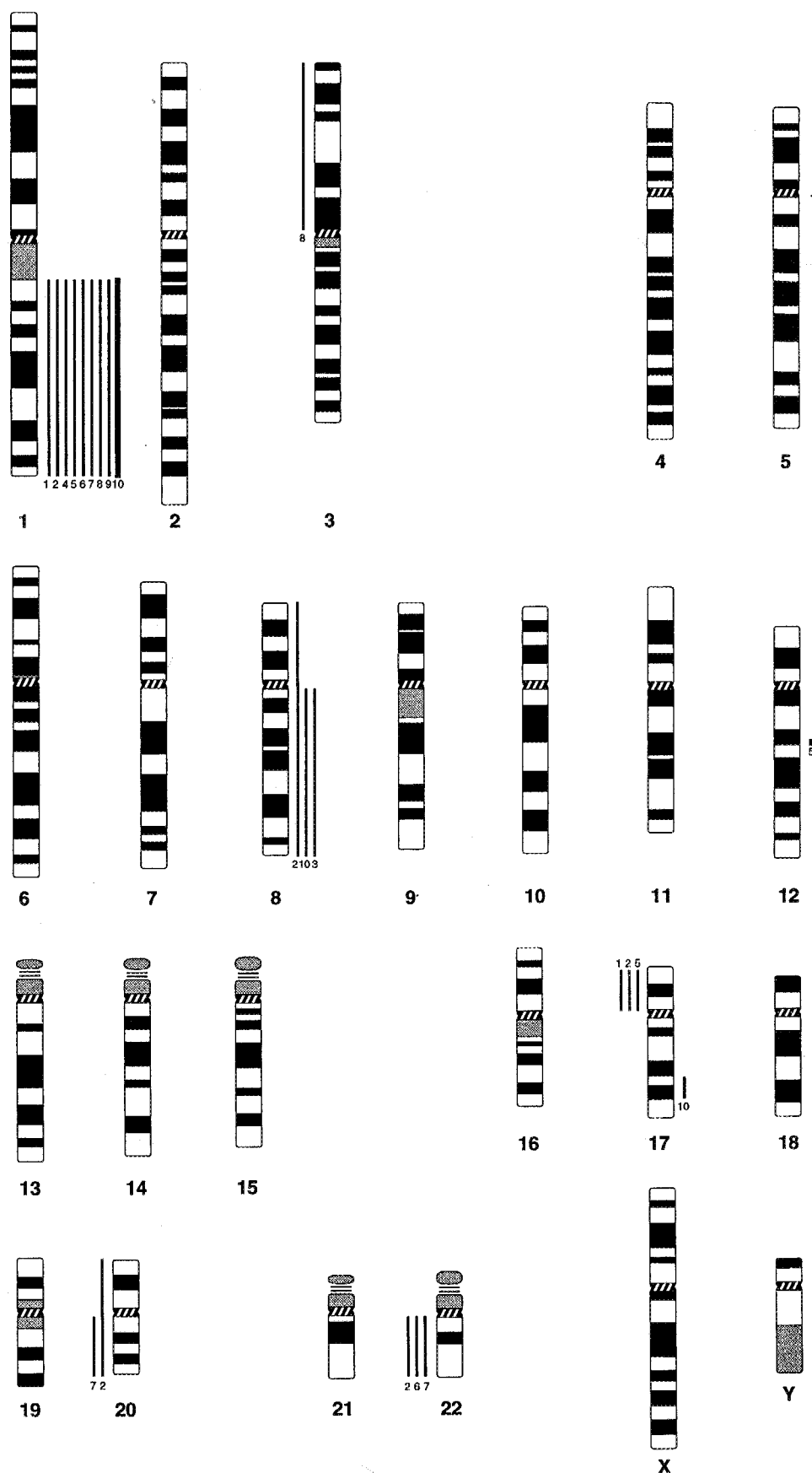
5419

Fig. 4. Summary of the genetic imbalances detected in 10 diploid primary breast carcinomas. *Vertical lines* on the *left* side of each chromosome ideogram represent a loss of genetic material in the tumor, whereas those on the *right* correspond to a gain. Changes in individual cases can be identified by the case numbers provided on each line. Amplification sites are represented as *solid squares* or *bars*.

Fig. 5. Summary of the genetic imbalances detected in 10 aneuploid primary breast carcinomas. *Vertical lines* on the *left* of each chromosome ideogram represent a loss of genetic material in the tumor, whereas those on the *right* correspond to a gain. Changes in individual cases can be identified by the case numbers provided on each line. Amplification sites are represented as *solid squares* or *bars*.
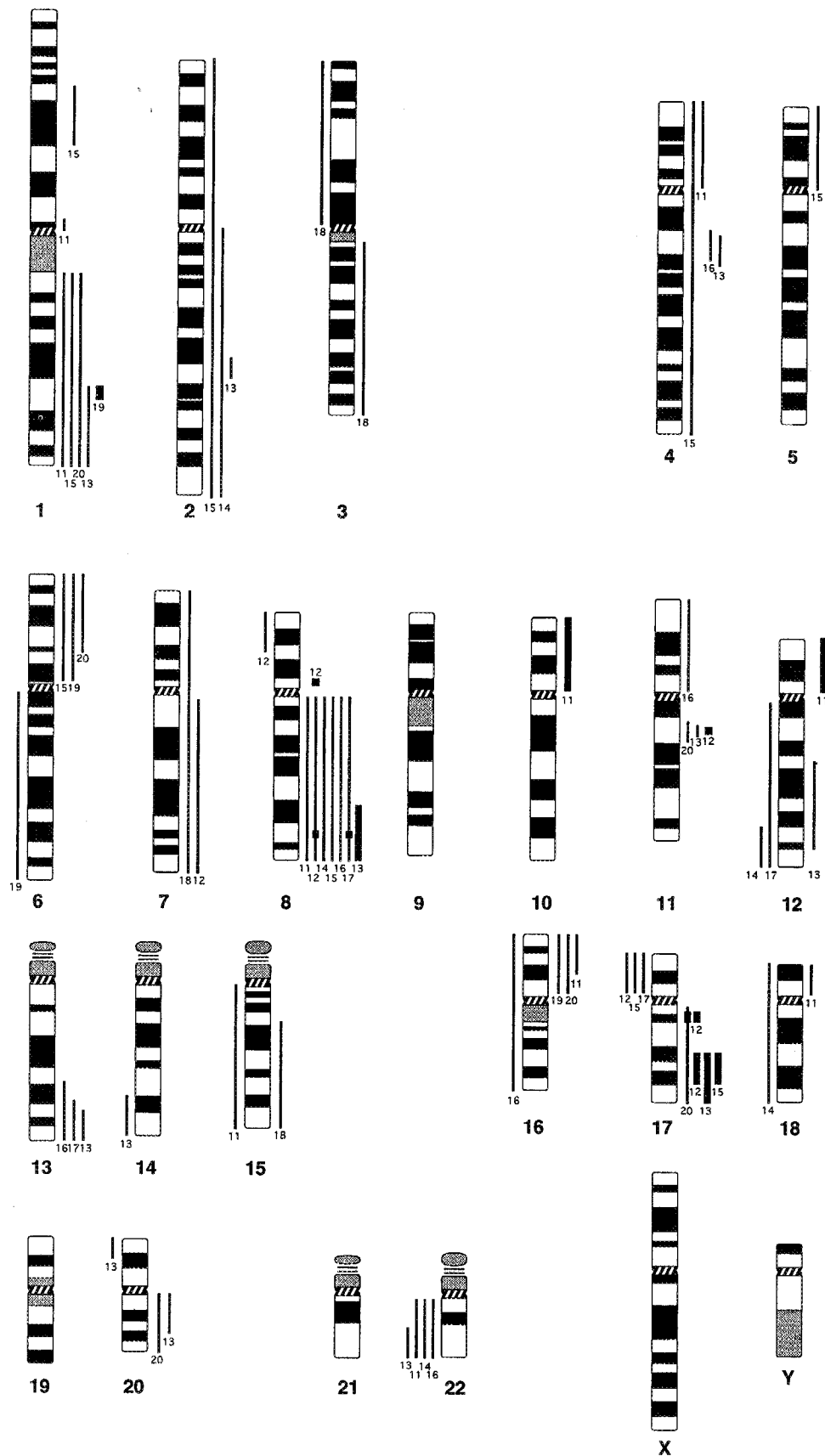
region harbors the tumor suppressor gene *NF2* (22q12.2), which is deleted or mutated in a variety of neurofibromatosis-related tumors (38). Whether the *NF2* gene fulfills a comparable function in breast tumors remains to be evaluated. In 1 of 10 diploid tumors, a high-level copy number increase was detected on chromosomal band 12q15 (case 5). This coincides with the chromosomal location of the *MDM2* protooncogene (39). In this tumor, the amplification of the *cyc*D1 gene that maps to chromosome band 11q13 was detected by Southern blot analysis. No increased fluorescence ratios, however, were detected on this chromosomal subregion after CGH, a failure that might possibly be due to limited sensitivity of CGH to detect low copy number increases of small amplicons. In this particular tumor, two amplifications are present. This might be indicative of an increased genetic instability. It will be interesting to evaluate whether this atypical pattern will influence the clinical course.

The genetic changes in aneuploid tumors are more complex and involve 19 different chromosomes. Surprisingly, the percentage of chromosome 1q gain is lower than in diploid tumors. This is in favor of the hypothesis that diploid and aneuploid breast tumors represent different entities. The most consistent gain is an overrepresentation of 8q, that is, in two cases accompanied by a localized high-level amplification of chromosome band 8q24, the location of c-*myc*. Next to the c-*erb*B-2 amplification on 17q11.2, which was observed twice, we mapped high copy number changes to chromosomal band 17q22–24. This observation is consistent with findings by Kallioniemi *et al.*, (9) who identified an increased copy number in this region in a high percentage as well. Similar to the findings in diploid tumors, a recurrent loss of genetic material was mapped on chromosomes 17p and 22q, a finding that underlines the importance of 22q loss in the genesis of breast carcinomas. Interestingly, no underrepresentation of chromosome 17p material was observed in cases 11 and 14. However, the detection of mutant *p53* expression in these tumors reveals an alternative mechanism of *p53* inactivation and underscores the importance of this gene in cell cycle control in breast epithelial cells.

The amplification of the protooncogenes c-*myc* (case 11), c-*erb*B-2 (case 12), and *cyc*D1 and *int*-2 (case 13) detected by means of Southern blot analysis was in accordance with data after CGH, when we observed increased fluorescence ratios on chromosomal regions 8q, 17q11.2–12, and 11q13, respectively. However, the amplification of c-*erb*B-2 in case 11 could not be confirmed by CGH. In cases 12, 13, and 17, increased fluorescence ratios were detected on chromosomal band 8q24 that were not accompanied by the amplification of the c-*myc* oncogene. This could be attributable to sampling errors in heterogeneous tumor samples or to the fact that the control gene was located on chromosome 8q as well (although some 15 Mb apart from c-*myc*); in these three cases the entire long arm of chromosome 8q was subject to copy number increases, which make it less likely to detect signal intensity variations between the control probe on chromosomal band 8q22 and the c-*myc* gene on 8q24 using Southern blot analysis. However, it could also indicate that the amplicon on 8q24 contains other genes that render a growth advantage to the cell when they are amplified.

We observed a clear difference in the frequency of copy number changes when fibroadenomas were compared with carcinomas of the breast. No copy number changes were observed in fibroadenomas. When diploid tumors are compared with aneuploid ones, the differences in both the frequency as well as the chromosomal distribution of numerical aberrations were obvious. Diploid tumors reveal few copy number changes that involve virtually exclusively the gain or loss of entire chromosomes or chromosomal arms, an observation that may indicate a segregation error as a primary event (40). Noteworthy is the observation that the cytogenetic correlate of the poor prognosis of patients suffering from aneuploid carcinomas seems to be a higher

number of chromosomal aberrations that also involve subchromosomal, regional, low and high copy number increases (amplifications), which could be the result of impaired cell cycle control and pronounced genetic instability.

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